Regulation of interleukin-18 by THP-1 monocytoid cells stimulated with HIV-1 and Nef viral protein

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ABSTRACT. Interleukin (IL)-18 is a proinflammatory cytokine that plays an important role in both innate and adaptive immune responses against several infectious pathogens. Relatively little is known about its production in HIV-1 infection, and there are controversial data on the influence of IL-18 on HIV-1 replication *in vitro*. In this study, we investigated the effect of HIV-1 infection, and challenge with recombinant HIV-1 proteins, on IL-18 production by THP-1 cells. This is a monocytoid cell line spontaneously producing IL-18, and consequently is particularly suitable for the study of HIV-1 effects on this type of cytokine regulation. The results reported here demonstrate a significant reduction in IL-18 secretion during HIV-infection. In fact, low levels of IL-18 were released until 120 h from viral challenge (15 ± 11pg/mL at 24 h and 17 ± 13 at 96 h and < 12.5 at 120 h), whereas IL-18 production by uninfected control cells was 193 ± 104pg/mL and 214 ± 114 pg/mL at 24 h and 120 h respectively. At 168 h of incubation, IL-18 production by infected and uninfected cells was found to be 164 ± 88 pg/mL and 325 ± 101 pg/mL respectively (p = 0.001). Of the following viral proteins: gp 120, p24 and Nef, only the last one induced decreased IL-18 secretion in the supernatants of THP-1 cells. This effect is more evident with the concentrations of 5 –1.25 µg/mL of Nef protein (p < 0.0001). In conclusion, our data show that HIV-1 and its regulatory protein, Nef, are able to down-regulate the release of IL-18, *in vitro*. These results confirm that a variety of modulating effects on the immune response, induced by HIV-infection, may facilitate progression of HIV-1 infection.

Keywords: IL-18, HIV-1 infection, Nef protein, THP-1 cells

IL-18 is a proinflammatory cytokine released by macrophages and, in cooperation with IL-12, it stimulates the production of interferon-gamma from effector Th1 cells; moreover, IL-18 regulates IL-2 production as well [1-3]. In co-operation with IL-12 and IL-23, IL-18 stimulates Th1-mediated immune responses, and it plays a critical role in the host defence against intracellular pathogens [3, 4], facilitating attempts to restore the Th1→ Th2 shift in HIV-positive subjects [5]. However, the role of IL-18 in viral infections is more controversial with respect to infection caused by intracellular microorganisms [4]. Co-infection with HIV and human Herpes virus (HHV)-8 [6] or with hepatitis C virus (HCV) [7] reduces the production of IL-18. High levels of circulating IL-18 in HIV-infected subjects have been demonstrated by various investigators [6, 8], although without unequivocal consensus [9]. Moreover, Wolday *et al.* [10] have shown that HIV-1 alters the T helper cytokines, IL-12 and IL-18 responses to the protozoan parasite *Leishmania donovani*. In addition, it has been reported that IL-18 stimulates production of transforming growth factor beta (TGF-beta), which may be responsible for suppression of several cytokines by peripheral blood mononuclear cells (PBMC) of HIV-infected patients [11]. In addition, Choi *et al.* [12] have demonstrated that IL-18 may inhibit HIV-1 production in PBMC through intermediate stimulation of IFN-gamma, and this inhibition is present during the early stages of viral infection and is associated with the reduction of HIV-1 receptor expression. However, other investigators found that IL-18 is able to enhance *in vitro* HIV-1 replication in monocytoid and T cells [13, 14]. He *et al.* [9] have shown that the production of IL-18 from PBMC, stimulated with *Staphylococcus aureus* Cowan strain 1, was significantly decreased in HIV-1 seropositive hemophiliacs. Finally, Chaisavaneeyakorn *et al.* [15] showed that HIV-1 infection or HIV/malaria co-infection decreases the IL-18 production from placental intravillous blood mononuclear cells of pregnant women.

THP-1 is a human monocytic-leukaemia cell line that supports replication of HIV-1 strains with known tropism for T lymphocytes [16]. In addition, THP-1 cell line constitutively produces IL-18 at detectable levels [17]. These cells represent a possible model of human cells, as sinusoidal leukocytes, which spontaneously produce cytokines. It is interesting to note that IL-18 has a relevant role in immune-surveillance [18].

Nef protein, a regulatory HIV-1 protein, is often endowed with inhibitory activity of host immune responses [19-21],

and can also be biologically active in exogenous form, as demonstrated in the U937 promonocytic cell line that may promote HIV-1 replication [22]. Exogenous Nef protein is also able to promote morphological and functional differentiation of immature dendritic cells [23]. Moreover, Pugliese *et al.* [19] showed that exogenous Nef protein upregulates expression of fibronectin receptor in lymphoid T cell lines (MT-4 and H9 T cells), whereas it downregulates expression of CD4 and CD71 receptors.

In our study, we evaluated the effects of HIV-1 infection or challenge with gp120, p24, and Nef viral protein on IL-18 production by THP-1 cells. A particular feature of this study (in contrast with the research of other authors that have investigated IL-18 production induced in immune cells) is the use of an *in vitro* model of cells that spontaneously secrete cytokines. This is in order to better simulate the conditions that occur *in vivo* as regards the natural and immediate defences of the host against infection [18].

MATERIALS AND METHODS

Cell line

THP-1 cell line employed in this study is a human, monocytic-leukaemia cell line, derived from blood culture from a 1 year-old Japanese child with leukaemia, at relapse, and firstly described by Tsuchiya *et al.* [24]. This cell line was kindly supplied by Dr. Beth Lee, Renal Division, Washington University Medical Centre, St. Louis, MO, USA.

H9-HTLV III_B cells (H9-V), a persistently HIV-1 infected, H9 T cell line, kindly provided by Dr. Robert C. Gallo (Baltimore, USA), was used to obtain the virus for THP-1 cell infection. It should be noted that HIV IIIB is also able to infect myeloid cells, such as embryonic astrocytes, producing expression of the *Nef* gene [25].

Cell cultures were kept in suspension in Nunc flasks (Nunc, Kamstrup, Denmark), in RPMI-1640 medium (Gibco Life Technologies, Paisley, Scotland), supplemented with 10% (v/v) foetal calf serum (FCS–Celbio, Milano, Italy), 200 µg/mL glutamine (Merck, Darmstadt, Germany) and 40 µg/mL gentamcin (Schering-Plough, Milano, Italy). All the cultures were maintained in a Heraeus incubator, thermostatically controlled at 37°C, in an atmosphere containing 5% CO_2 (v/v in humidified air).

Infection of THP-1 cells with HIV-1, and IL-18 titration

Stocks of HIV-1 used in the experiments reported here were obtained from the supernatants of H9-V cells, as previously described [26]. Viral stocks were titrated and standardized using a biological method based on CPE evaluation, and, in particular, the 50% endpoint method [27]. The viral type is consequently IIIB

THP-1 cells were suspended at 5 x 10^5/mL. After centrifugation for 10 min. at 140 x *g* in 50 mL centrifuge tubes (Nunc), cellular pellets were infected with 50 $CCID_{50}$/1 x 10^6 cells of an HIV-1 stock suspension in RPMI 1640 plus 2% FCS ($CCID_{50}$ = Cell Cultures 50% Infecting Dose). Control cells were mock-infected with RPMI 1640 plus 2% FCS without the virus. The cells were then incubated at 37°C for 1.5 h and subsequently washed three times with PBS by centrifugation at 140 x *g*, suspended in RPMI-1640 plus 10% FCS at a concentration of 2.5 x 10^5/mL and incubated at 37°C in a 5% CO_2/ 95% humidified air atmosphere.

Supernatants were collected for IL-18 titration at different times from infection (24, 48, 96, 120, 168 hours). In parallel, uninfected control cells were also analyzed. Moreover, in some experiments, IL-18 levels were also determined in the cryolysates obtained by freezing and thawing the cultures in order to detect all the cytokine produced, or for evaluating only the intracellular levels corresponding to the difference between total production and supernatant content. IL-18 titration was effected using an immunoenzymatic system (Human IL-18 ELISA Kit, MBL Medical & Biological Laboratories CO., Ltd, Naka-ku Nagoya, Japan), in accordance with the suggestions of the manufacturer. The sensitivity of the assay was 12.5 pg/mL.

Evaluation of the effect of some HIV-1 proteins, and of two bacterial products on IL-18 production by THP-1 cells

Recombinant gp120, p24 (of HIV-1 IIIB) and Nef proteins from *Escherichia coli* were purchased from Intracel Corporation (Cambridge, MA, USA). LPS from *E. coli* (O26 B6) was purchased from Sigma Chemichals (St. Louis, MO, USA). *Proteus* extract was kindly supplied by Roussel Maestretti (Milano, Italia). THP-1 cells were incubated with different doses of the previously described proteins, and 24 hours later, the supernatants, and, in some cases the cryolysates too, were collected for IL-18 titration.

Statistical evaluation of the data

Data are expressed as mean and standard deviation. Student's *t*-test was used to detect significant differences between the means of two groups of data. Moreover, differences among various groups were statistically evaluated by the ANOVA test. Each test was performed at least in triplicate and repeated twice. Results were considered significant when $p < 0.05$.

RESULTS

In a first set of experiments, we studied the effect of HIV-1 infection on IL-18 production by THP-1 cells, at different incubation times.

As can be seen in *table 1*, in the supernatant of infected cells, we detected low levels of IL-18 in the early days of infection (15 ± 11pg/mL and 17 ± 13 pg/mL of the cytokine, respectively at 1 and 4 days after infection), whereas IL-18 levels increased sharply after 7 days.

In contrast, in the supernatant of the control cells, we found increased levels of IL-18 from the first days of infection (193 ± 104, 130 ± 94 and 325 ± 101 pg/mL of IL-18, respectively after 1, 4 and 7 days; $p < 0.01$ by ANOVA test).

Subsequently, in a further set of experiments, we evaluated the effects of some proteins of HIV-1, including Nef, gp120 and p24, on IL-18 production by THP-1. IL-18 detection was performed after 24 h from incubation, either in the supernatants or in the cryolysates, in order to increase the total levels of IL-18.

Table 1
Secretion of IL-18 in the supernatant of TPH-1 cells challenged with HIV-1, and in the controls cells

Time (days)	IL-18 (pg/mL)	
	Uninfected (TPH-1) cells	Infected (TPH-1) cells
1	193 ± 104	15 ± 11
4	130 ± 94	17 ± 13
5	214 ± 114	ND
7	325 ± 101	164 ± 88*

* $p < 0.01$ evaluated with ANOVA test. ND = not detectable. Moreover, evaluating the values of 1 and 4 days together, the differences between the means of infected cells and controls were significant for $p = 0.01$ using Student's *t*-test.

The results regarding Nef protein are reported in *table 2*. Significant differences between the groups were detected in the supernatants by the ANOVA test ($p = 0.001$). Moreover, a significant reduction in IL-18 levels, as compared the controls (Student's *t*-test), was found in the supernatants at concentrations of 5 µg/mL, 2.5 µg/mL and 1.25 µg/mL of Nef. In particular, at a concentration of Nef of 1.25 µg/mL, the lowest, significant, active dose, IL-18 levels in the supernatant were respectively 57.4 ± 8.5 pg/mL in the cells treated with Nef and 184 ± 48.4 pg/mL in the control cells ($p = 0.01$). In addition, in the cryolysates no significant differences were found among the different samples (ANOVA test). However, slight reductions as compared to the controls were shown at concentrations of 5 and 2.5 µg/mL of Nef, with a significant difference, evaluated by Student's *t* test, of $p = 0.023$ at the dose of 5 µg/mL of Nef and $p = 0.031$ at 2.5 µg/mL. In addition, evaluating only the intracellular (difference between cryolysate levels and extracellularly-released cytokine), levels of IL-18 produced in the presence of 5 µg/mL of Nef, the mean content of IL-18 was found to be significantly higher than in the control cells (252.5 ± 24.5 *versus* 128 ± 12.7; $p < 0.01$). This also occurred with the doses of 2.5 and 1.25 µg/mL of Nef protein (respectively 211.3 ± 11.6 *versus* 128 ± 12.7 with $p = 0.027$ and 246.6 ± 10.5 *versus* 128 ± 12.7 with $p = 0.013$).
Finally, pretreatment of Nef with anti-Nef monoclonal antibodies (Intracel) suppressed the inhibitory effect of the viral protein on IL-18 release (unpublished data).
Table 3 illustrates IL-18 production in the cryolysates of THP-1 cells incubated with p24 and gp120. The results obtained demonstrate that these viral proteins do not influence the production of IL-18, by THP-1 cells even at high concentrations. Moreover, IL-18 release into the supernatants of THP-1 cells incubated with p24 and gp120, did not show significant differences between the treated and the control cells.
In a subsequent experiment we evaluated, as a control test, the effect of two bacterial proteins on IL-18 production by THP-1 cells.
As shown in *table 4*, treatment with bacterial products, *Proteus* extract and *E. coli* LPSleads to a high level of production of IL-18 from THP-1 cells.
Finally, in order to determine the presence of IL-18 inhibitors in the supernatants of THP-1 cells infected with HIV-1, we incubated IL-18 (100 pg/mL – MBL immunoenzymatic test standard) at 4°C for 2 h, with the same supernatants (filtered through 100 KdA filters – Millipore, Kogyo KK, Japan), collected after 24 and 48 hours from the challenge. There were no significant differences in the IL-18 titres, and similar results were obtained with the supernatants of THP-1 cells treated with 10 or 5 µg/mL of Nef protein.

Table 2
IL-18 production by THP-1 cells not incubated or incubated with different concentrations of HIV-1 Nef proteins evaluated in the supernatants, and in the cryolysates, 24 hours after the challenge

Nef (µg/mL)	IL-18 (pg/mL)	
	Surnatants	Cryolysates
5	27.9 ± 2.3*	280.4 ± 27.2**
2.5	41.9 ± 5.6	253.2 ± 17.2
1.25	57.4 ± 8.5	304 ± 19
0.62	168.8 ± 53.4	376.4 ± 92
0.31	167.8 ± 28.6	377 ± 99
0	184 ± 48.4	312 ± 35.7

*$p = 0.001$ (supernatants) and ** $p = 0.124$ (cryolysates) – ANOVA test. Supernatants *versus* controls = $p \leq 0.01$ until the dose of 1.25 µg/mL of Nef; Cryolysates *versus* controls = NS in all the cases – Student's *t*-test. Supernatants *versus* cryolysates = $p < 0.001$ until the dose of 1.25 µg/mL – Student's *t*-test.

Table 3
IL-18 production by THP-1 cells not incubated or incubated with different concentrations of HIV-1 structural proteins p24 or gp120, evaluated in the cryolysates, 24 hours after the challenge

Concentration (µg/mL)	IL-18 (pg/mL)	
	p24	gp120
10	340.5 ± 59.5[a]	348 ± 88[a]
5	405.5 ± 132.5	388 ± 113
2.5	340.5 ± 59.5	285.7 ± 12.7
1.25	295 ± 33	313.6 ± 36.6
0.62	341.3 ± 46.3	286.3 ± 27.3
0	354.8 ± 58	343.2 ± 83.2

[a] ANOVA test = not significant.

DISCUSSION

In this study, we have demonstrated that HIV-1 infection and Nef protein may reduce the release of IL-18 from THP-1 cells, a monocytoid cell line spontaneously producing this cytokine. In fact, Nef is a regulatory HIV-1 protein endowed with pleiotropic effects directed either to the cellular regulation of HIV-replication or to the inhibition of immune responses against HIV-1 infection [28-30]. In addition, Nef protein impairs induction of Th1 cytokines and down-regulates cytokine production by HIV-1-specific CD8+T cells [31]. IL-18 is an interesting

Table 4
IL-18 production by THP-1 cells not incubated or incubated with different doses of *Proteus* extract or *E. coli* LPS, evaluated in the cryolysates, 24 hours after the challenge

Concentration (µg/mL)	IL-18 (pg/mL)	
	Proteus	*E. coli* LPS
5	467.6 ± 63*	497.4 ± 133.6**
2.5	626.6 ± 84.5	278.8 ± 31.3
1.25	447 ± 133	264.3 ± 19.5
0	309 ± 80.7	302.9 ± 19

*$p = 0.02$ and ** $p = 0.01$ (ANOVA test).

proinflammatory cytokine in HIV-infection, inasmuch as it has multiple biological functions, and, in particular, it is endowed with an important role in T helper lymphocyte activation [1, 2]. In fact, this is a unique cytokine that stimulates both Th1 and Th2 responses in different conditions [32]. Moreover, the THP-1 cells, employed in our study, represent a possible experimental model of immune cells spontaneously producing cytokines during an innate immune response [18].

Although several studies have investigated the role of IL-18 in HIV-1 infection, its effects on the immune response during the disease remain controversial, and, in part, somewhat contradictory. It seems that IL-18 acts either as a positive effector in the host's response against HIV-1 replication, or as a proinflammatory agent induced by HIV that plays a role against the host [5, 14]. The elevated circulating levels of IL-18, found particularly in the advanced stage of the disease both in pediatric and adults patients [5, 8, 33], seem to enhance HIV-1 replication in monocyte and T cells [13, 14]. However, some investigators have observed that circulating levels of IL-18 might not reflect the biological activity of IL-18, inasmuch as the IL-18 binding protein directly binds IL-18, and specifically neutralizes the biological activity of this cytokine [13, 34, 37, 38].

Nevertheless, He *et al.* [9] have shown that the production of IL-18 by PBMC stimulated with *Staphylococcus aureus* Cowan strain 1 was significantly decreased in HIV-1 seropositive hemophiliacs. In addition Chaisavaneeyakorn *et al.* [15] have also demonstrated that HIV-1 infection or HIV/malaria co-infection decrease IL-18 production by placental intravillous blood mononuclear cells from pregnant women.

It has been postulated that down-regulation of IL-18 expression could depend, in part, on IL-18-induced overproduction by TGF-β [11]. In fact, Ahmad *et al.* [8] have suggested that TGF-β overproduction seems to be responsible of the inhibition of IL-18 production from PBMC from HIV-infected patients. However, in our study, we found that THP-1 cells treated with Gram-negative LPS produce high levels of IL-18, and we recall that some bacterial products, including LPS, are able to induce high levels of TGF-β [35].

The early inhibitory effect of HIV challenge on IL-18 release by THP-1 cultures found in our study, could depend on acute phenomena produced by high doses of the virus employed, with a consequent shift of the balance between cellular metabolism and viral replication. In addition, the inhibitory effect of IL-18 induced by Nef, especially on the extracellular environment, as observed in our experiments, seems to suggest a possible activity of the viral protein in this phenomenon.

In fact, no soluble, IL-18 inhibitors were found in our experiments, and the intracellular content of IL-18 in THP-1 cells treated with Nef is significantly higher than in the control cells. These data suggest inhibition of Nef release, rather than an effect on cytokine production.

A new hypothesis on the IL-18 regulatory effect of HIV-1 is related to the induction of inflammasomes. In fact, it has been demonstrated that several inhibiting caspase-1 inflammatory proteins, may reduce the processing of IL-1β precursor, and consequently the release of this cytokine [38, 39]. It is conceivable to think that this could also happen for IL-18. In fact, a strict correlation exists between IL-1β and IL-18, inasmuch as these two cytokines belong to the IL-1 family and both require proteolytic processing by caspase-1 for their activation [40].

In addition, Olivetta *et al.* [41] observed a lack of secretion of IL-1α, IL-12 and IL-18 after an apparent increase in gene transcription induced by exogenous Nef protein. This could depend on a transport defect for these cytokines mediated by Nef [36]. Thus, we may postulate that Nef protein induces a defect in the intracellular transport of IL-18, or in the expression of cellular receptors involved in the mechanisms involved in IL-18 release. These mechanisms were proposed for various cellular proteins of a receptorial nature [42, 43]. Moreover, Nef protein may also interact with proteins involved in actin cytoskeleton rearrangement, perhaps inhibiting or retarding some phenomena of intracellular transport mechanisms [44]. In fact, our data demonstrate a significant reduction in IL-18, particularly in the supernatants of THP-1 cells treated with Nef or infected with HIV-1.

Finally, in our study, HIV-1 proteins, gp120 and p24, were not able to modulate IL-18 production from THP-1 cells.

In conclusion, the down-regulatory activity of HIV-1 and Nef protein on IL-18 production, along with increased HIV-1 replication stimulated by IL-18 may suggest that HIV-1 has evolved strategies to escape host immune defences through activation or inhibition of several cytokines and chemokines. However, additional investigations are needed to determine the exact role of IL-18 in HIV-1 infection, and the mechanisms involved in the modulation of this cytokine.

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